Computer-based analysis of *Haemophilus parasuis* protein fingerprints

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Abstract

The present study aimed to compare the whole-cell protein profiles of *Haemophilus parasuis* field isolates by using a computer-based analysis, and evaluate the relationship between polyacrylamide gel electrophoresis (PAGE) type and virulence potential based on isolation site. A dendrogram clustering isolates with similar protein profiles was generated. *Haemophilus parasuis* isolates were grouped into 2 major PAGE type groups. The PAGE type II isolates were characterized by the presence of major proteins with molecular weights varying from between 36 and 38 kDa and included 90.7% of the isolates recovered from systemic sites, such as pleura, pericardium, peritoneum, lymph nodes, joints, and brain. Isolates classified as PAGE type I were characterized by the absence of this group of proteins and included 83.4% of the isolates recovered from the upper respiratory tract of healthy animals. The present study further corroborates the existence of a unique group of major proteins in potentially virulent *H. parasuis* isolates.

Résumé

Dans cette étude, une comparaison a été effectuée entre les profils de protéines des cellules entières d'isolats cliniques de Haemophilus parasuis *par analyse informatique, ainsi que d'évaluer la relation entre les patrons électrophorétiques obtenus sur gel de polyacrylamide (PAGE) et le potentiel de virulence basé sur le site d'isolement. Un dendrogramme regroupant les isolats ayant des profils de protéines similaires a été produit. Les isolats d'*H. parasuis *ont été regroupés en 2 groupes majeurs basés sur leur profil PAGE. Les isolats appartenant au profil de type II étaient caractérisés par la présence de protéines majeures avec des poids moléculaires variant entre 36 et 38 kDa et comptait 90,7 % des isolats retrouvés des sites d'infection systémique tels que la plèvre, le péricarde, le péritoine, les nœuds lymphatiques, les articulations et le cerveau. Les isolats classées comme appartenant au profil de type I étaient caractérisés par l'absence de ce groupe de protéines et comprenait 83,5 % des isolats provenant du tractus respiratoire supérieur d'animaux en santé. Cette étude corrobore l'existence d'un groupe unique de protéines majeures parmi les isolats potentiellement virulents de* H. parasuis.

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Haemophilus parasuis is a commensal organism of the upper respiratory tract of pigs that can invade the host and cause severe systemic disease characterized by fibrinous arthritis, polyserositis, and meningitis (1). The factors involved in systemic invasion by *H. parasuis* are still unknown (2). Although no virulence factors have been established for *H. parasuis*, some virulence markers have been proposed. Nicolet et al (3) first described the presence of a unique 37 kDa protein expressed by *H. parasuis* isolates recovered from classical Glässer's disease cases. These isolates were classified as polyacrylamide gel electrophoresis (PAGE) type II, while respiratory isolates that lacked the 37 kDa protein were classified as PAGE type I (3). These results were later corroborated by Morozumi and Nicolet (4) and Morikoshi et al (5), who also reported that *H. parasuis* strains that induced an acute course of disease in field cases, such as septicemia, belonged to the PAGE type II group.

Rosner et al (6) and Kielstein et al (7) used a different system for the classification of *H. parasuis* whole-cell protein profiles. Seven PAGE types were described based on the major proteins observed: type I (29, 37, 39 and 51 kDa), type IIa (27 and 45 kDa), type IIb (47 kDa), type IIIa (45 kDa), type IIIb (27 and 39 kDa), type IV (32 and 51 kDa), and type V (39 and 45 kDa). Virulent isolates were distributed in PAGE types I, IIa, IIb, IIIa, and IIIb. Non-virulent isolates were either PAGE types IIa, IV, or V.

Rapp-Gabrielson et al (8) examined *H. parasuis* field isolates and reference strains by serotyping, sodium dodecyl sulphate (SDS) PAGE analysis of outer membrane proteins (OMPs), and multilocus enzyme electrophoresis (MLEE). The SDS-PAGE analysis of OMPs indicated that in 78% of the isolates, including all isolates from pigs with polyserositis, the major protein migrated with an apparent molecular weight of 37 to 38.5 kDa. Similar results were obtained by Ruiz et al (9), who demonstrated that *H. parasuis* isolates recovered from systemic sites were characterized by the presence of a major OMP with molecular weight of 36.6 to 38.5 kDa.

Results obtained by previous studies strongly suggest a clonal relationship between *H. parasuis* isolates capable of causing systemic disease. However, the association between the presence of a specific protein and virulence is still controversial due to visual inspection of complex protein patterns and variability among studies regarding the characterization of PAGE types. The purpose of this investigation was to characterize and compare the whole-cell protein profiles of

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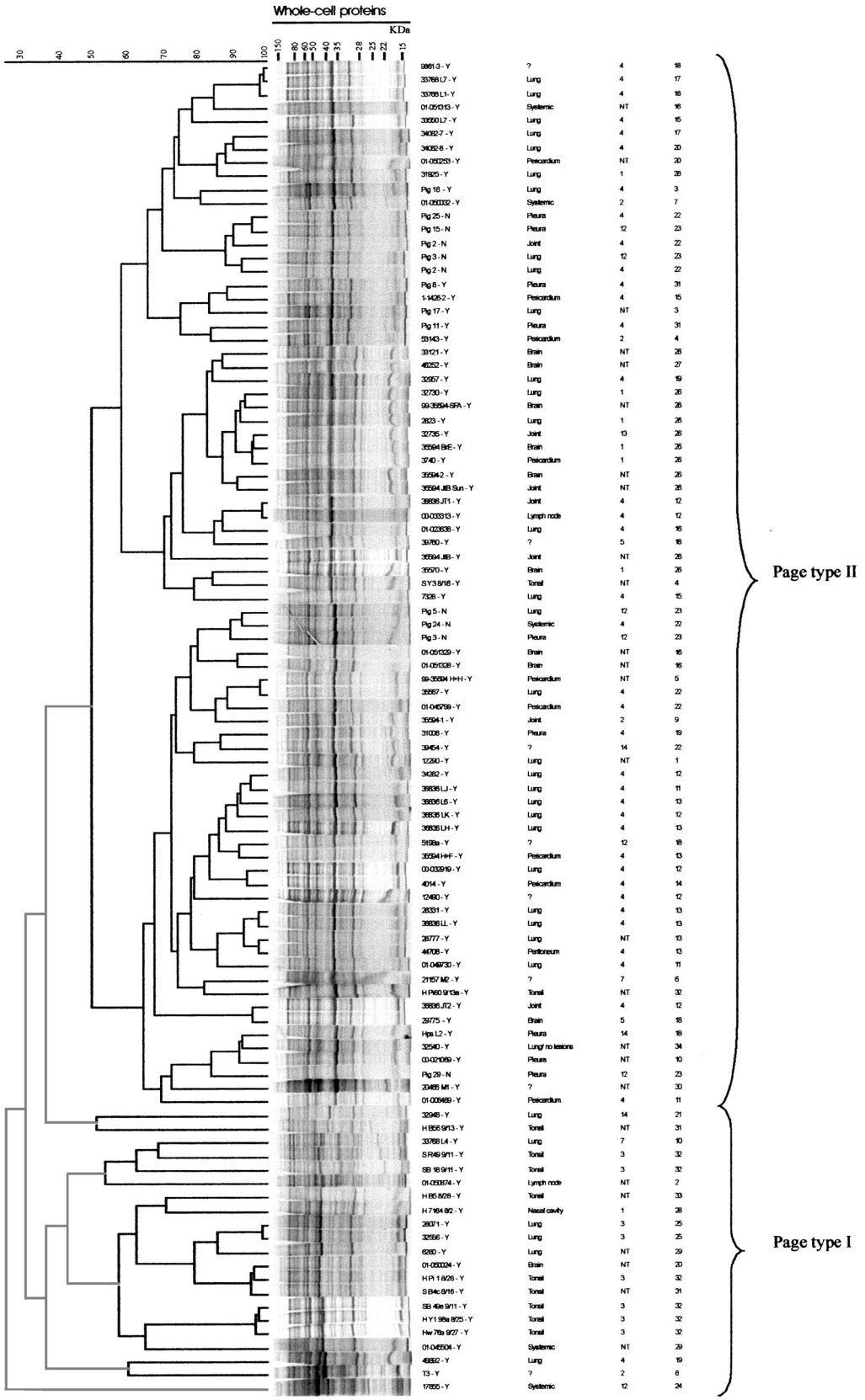

Figure 1. Cluster analysis of *Haemophilus parasuis* field isolates based on whole-cell protein profiles (sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE], Pearson correlation, UPMGA). The scale indicates the percent of similarity. The columns in the figure indicate the isolate identification, site of isolation, serotype and genotype groups, as defined by Oliveira et al (10). Relevant clusters of strains are shown by gray lines. Clusters were defined based on the calculation of the point-bisectional correlation (BioNumerics).

Table I. Characterization of *Haemophilus parasuis* polyacrylamide gel electrophoresis (PAGE) groups based on major proteins molecular weight, isolation site, serovar, and genotype groups

Cluster	Major proteins (kDa)	Isolation sites[a] (*n*)	Serovar groups[a] (*n*)	Genotype groups[a] (*n*)	PAGE type
1	36–38	Lung (29)	1, 12 (6 each)	1, 5, 6, 7, 9, 10, 14, 27, 28, 30,	II
		Systemic[b] (39)	2 (3)	32, 34 (1 each)	
		URT (2)	4 (37)	3, 4, 17, 19, 20, 31 (2 each)	
		Unknown (7)	5, 14 (2 each)	11, 15 (3 each)	
			7, 13 (1 each)	16 (4)	
			NT (19)	23 (5)	
				18 (6)	
				12, 13, 22 (7 each)	
				26 (11)	
2	39–40.5	Lung (1)	14, NT (1 each)	21, 31 (1 each)	I
		URT (1)			
3	43–46	Lung (1)	3 (2)	2, 10, 32 (1 each)	I
		Systemic (1)	7, NT (1 each)		
		URT (2)			
4	44–46	Lung (3)	1 (1)	20, 28, 30, 33 (1 each)	I
		Systemic (2)	3 (6)	25, 29 (2 each)	
		URT (7)	NT (5)	32 (4)	
5	42	Lung (1)	2, 4 (1 each)	8, 19 (1 each)	I
		Unknown (1)			
6	47	Systemic (1)	12 (1)	24 (1)	I

URT — Upper respiratory tract (tonsil or nasal cavity); *n* — Number of isolates per group (isolation site, serovar, or genotype); NT — non-typeable

[a] Data obtained from Oliveira et al (10)

[b] Brain, pleura, pericardium, joint, peritoneum

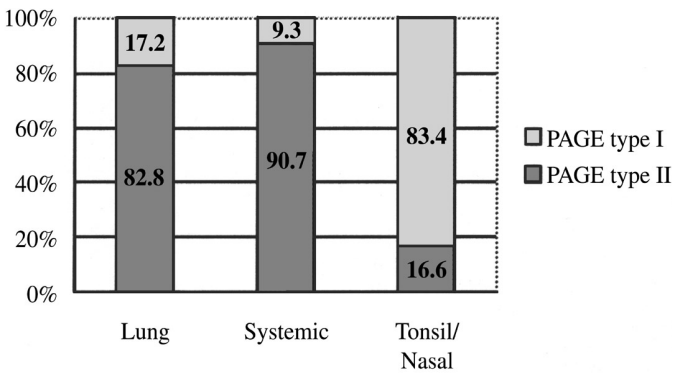

Figure 2. Distribution of *Haemophilus parasuis* field isolates according to polyacrylamide gel electrophoresis (PAGE) type and site of isolation.

98 *H. parasuis* field isolates recovered from respiratory and systemic sites using computer-based analysis. In addition, a universal scheme for PAGE type classification is proposed based on the concept previously described by Nicole et al (3).

Ninety-eight *H. parasuis* field isolates obtained from the Veterinary Diagnostic Laboratory at the University of Minnesota were used for whole-cell protein evaluation. Isolate identification, site of isolation, serovar and genotype groups, all reported in a previous study (10), are shown in Figure 1. *Haemophilus parasuis* was cultured overnight at 37°C in sheep blood agar with a *Staphylococcus aureus* nurse streak to check purity. Pure *H. parasuis* cultures were transferred to chocolate agar plates and incubated overnight at 37°C in a candle jar. Bacterial growth was harvested from chocolate agar plates using sterile phosphate buffered saline (pH 7.4) and used for protein quantification and SDS-PAGE. The protein contents of each bacterial suspension were determined by protein microplate assay (DC Protein microplate assay; Bio-Rad, Hercules, California, USA), as instructed by the manufacturer. Bovine serum albumin was used to produce a standard protein curve, with protein concentrations varying from 0 to 2 mg/mL. After quantification, bacterial suspensions were adjusted to a final protein concentration of 1 mg/mL. Whole-cell protein extracts and SDS solubilization were performed as described previously, with some modifications (3). Briefly, 40 μL of the bacterial suspension containing 1 mg/mL of proteins were mixed with 60 μL of a solution containing 0.5 mM Tri-HCl, pH 6.8; 25% glycerol; 2% SDS; 0.01% bromophenol blue; and 5% β-mercaptoethanol (Laemmli sample buffer; Bio-Rad). The suspension was boiled for 5 min. Fifty microliters of the suspension containing the whole-cell proteins were loaded into a 10% Tris-HCl gel (Protean II ready Gel Precast; Bio-Rad) and run at 100 mA in 1 × Tris-glycine-SDS buffer (Bio-Rad) for 3 h (11). A protein standard (Bio-Rad) was loaded into the 1st, 10th, and 20th lanes for proper alignment during computer analysis. Gels were stained using Coomassie brilliant blue (Bio-Rad). After staining was complete, gels were dried and scanned. The PAGE types were defined based on the previous description by Nicolet et al (3), with some modifications. Isolates containing major proteins weighing between 36 and 38 kDa, which included the previously described 37 kDa protein, were classified as PAGE type II. Isolates lacking this group of proteins were classified as PAGE type I. Gel

images were stored in disks as TIF files and analyzed using software (BioNumerics, version 2.5; Applied Maths, Kortrijk, Belgium). The whole-cell protein profiles obtained were analyzed by calculating the similarity matrices of whole densitometric curves of the gel tracks using the pair-wise Pearson's product-moment correlation coefficient. The protein profiles from 21.5 to 97.4 kDa were compared. Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages (UPGMA). A dendrogram containing all 98 *H. parasuis* isolates was constructed in order to assess the overall phenotypic diversity. Relevant clusters were defined using the 'cluster cutoff method' and the 'point-bisectional correlation' functions in the computer software.

Six clusters of isolates were observed based on the obtained whole-cell protein profiles (Table I, Figure 1). Cluster 1 included a total of 77 isolates and was characterized by the presence of major proteins with molecular weights varying from 36 to 38 kDa (PAGE type II). This cluster included the majority of isolates recovered from affected animals, including 29 isolates from pneumonic lungs and 39 isolates from systemic sites, such as pericardium, pleura, joint, brain, and lymph node. Clusters 2 to 6 included a total of 21 isolates and were characterized by the absence of these major proteins (PAGE type I). These clusters included 10 isolates recovered from the upper respiratory tract of healthy animals, plus 6 isolates recovered from pneumonic lungs and 4 isolates from systemic sites (Table I). The association between PAGE type and site of isolation is shown in Figure 2.

Serovar and genotype groups included in clusters 1 to 6 are shown in Table I. The PAGE type II isolates included serovars 1, 2, 4, 5, 7, 12, 13, 14, and non-typeable (NT) isolates. A high genetic diversity was observed in this group, with 27 different fingerprints patterns being identified. The PAGE type I isolates included most of the serovars observed in the PAGE type II group, with the exception of serovar 3, which was only observed in the PAGE type I group. This group also showed a high genetic diversity, with 14 different fingerprint patterns being identified.

This study further corroborates the existence of a unique group of major proteins in *H. parasuis* isolates recovered from affected animals, which is absent in isolates recovered from the upper respiratory tract of healthy animals. The definition of PAGE types and the association between protein profiles and virulence is not consistent in the literature. Nicolet et al (3) and Morozumi and Nicolet (4) demonstrated that isolates showing a 37 kDa protein (PAGE type II) were predominantly recovered from animals showing classical Glässer's disease lesions, such as fibrinous polyserositis, arthritis, and meningitis. A more detailed classification of *H. parasuis* whole-cell protein profiles was proposed by Rosner et al (6) and Kielstein et al (7), who suggested that virulent isolates were characterized by the presence of a 39 kDa protein and were classified as PAGE type I, while non-virulent isolates were characterized by the presence of a 45 kDa major protein and were mainly from PAGE types IV and V. Based on our findings, we believe that the PAGE type classification previously proposed by Nicolet et al (3) provides a better differentiation between potentially virulent and non-virulent *H. parasuis* isolates. However, results obtained in the present study suggest that the protein group characteristic of PAGE type II isolates can vary from between 36 and 38 kDa. This difference may be due to the larger number of isolates evaluated in this study (12 isolates from the upper respiratory tract, 35 isolates from pneumonic lungs and 43 isolates from systemic sites), in addition to the use of an accurate computer based-analysis of the obtained protein profiles. Nicolet et al (3) evaluated 2 isolates recovered from pneumonia cases and 6 isolates obtained from Glässer's disease cases.

A few exceptions were observed in the distribution of *H. parasuis* isolates in PAGE type groups. Two tonsil isolates were classified as PAGE type II and 4 systemic isolates were classified as PAGE type I. Similar results were observed by Morozumi and Nicolet (4) and Morikoshi et al (5), who demonstrated that *H. parasuis* isolates recovered from respiratory sites can be either PAGE types I or II, while systemic isolates are mainly from PAGE type II. Nicolet et al (3) suggested that the presence of the 37 kDa protein is a marker for isolates recovered from classical Glässer's disease cases, and that isolates recovered from respiratory sites, including pneumonic lungs, lack this group of proteins. However, results obtained in the present study showed that 82.8% of the isolates recovered from pneumonic lungs were classified as PAGE type II. This suggests that isolates from pneumonic lungs should be considered virulent, in contrast to isolates from normal lungs or upper respiratory sites.

Haemophilus parasuis isolates recovered from affected animals (pneumonic lungs or systemic lesions) showed a uniform whole-cell protein profile (cluster 1). Conversely, isolates recovered from the upper respiratory tract of healthy animals showed a higher degree of variability, which resulted in the distribution of these isolates into 5 different clusters. These results are in accordance with previous report by Ruiz et al (9), who also demonstrated that *H. parasuis* isolates recovered from the respiratory tract of healthy animals are highly variable regarding protein and genotype profiles.

No clear association between PAGE type and serovar or genotype groups was observed in the present study. Both PAGE types I and II showed a high genetic diversity and a broad variety of serovars. Similar results were reported by Rapp-Gabrielson et al (8), who did not find a clear association between protein profiles, serovar and MLEE patterns of *H. parasuis* field isolates. *Haemophilus parasuis* isolates from serovar 3, however, were mainly classified as PAGE type I and isolated from the upper respiratory tract of healthy animals, which suggests that these isolates have low virulence.

Although no conclusions can be drawn regarding the direct association of the 36 to 38 kDa major proteins and virulence of an isolate, these proteins can be considered virulence markers for *H. parasuis* isolates. These markers may be important for the development of broad-spectrum vaccines or diagnostic tests. However, further studies are necessary in order to better characterize the antigenic and cross-protective properties of the major proteins expressed by potentially virulent *H. parasuis* strains.

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